acetyl-cysteinyl and with OPA/*N*-L(D)-isobutyrylcysteinyl AA derivatives gave excellent resolution of enantiomers. Consequently, the CDR technique is the primary importance in a number of practical applications of the separation of enantiomeric AAs. The interaction of AAs with the enantiomerically pure reagents takes place at ambient temperature, without racemization, resulting in the formation of stable diastereomer derivatives.

Online LC-MS

In the case of AAs, thermospray ionization has been displaced by the milder techniques of electrospray (ES) and atmospheric pressure chemical ionization (APCI), converting analyte molecules without fragmentation into ions. The analyte should contain the AAs in a stable form: either in the free condition or in the form of stable derivatives, such as phenylthiohydantoins (PTH) or PTCs. Significantly reduced flow rates are essential (100–300 nL min^{-1}) for stable ES and APCI operation. In automated Edman microsequencing, the ES-MS of PTH derivatives. The protonated molecules were measured with a linear response in the 50–1000 fmol level.

Future Trends

Efforts are needed to extend the life time, plate number and reproducibility of columns, and to standardize testing methods. The extended use of thermostated columns is desirable in order to obtain reproducibility in absolute and relative retention times. LC-MS will be more widely used in laboratories as the cost of these instruments falls to the level of GC-MS, and/or an all-purpose interface becomes available.

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See also: **II/Chromatography: Liquid:** Derivatization; Mechanisms: Reversed Phase.

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Thin-Layer (Planar) Chromatography

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Introduction

Thin-layer chromatography (TLC) is a simple and inexpensive technique permitting a number of samples to be handled simultaneously, thus yielding a higher precision than sequential analysis. The inert character of the thin-layer material makes it ideally suitable for use with strong corrosive reagents and one can perform many kinds of chemical reactions on the plate, both from the points of view of detecting and locating the spot and of achieving improved separation. Certain groups of interest can be chemically bonded to the reactive groups of support material, e.g. silanization for reversed-phase studies. Impregnation of the adsorbent with a variety of reagents adds an additional feature for influencing the adsorption characteristics without covalently affecting the inert character of the adsorbent. TLC is also successful in providing direct resolution of enantiomers of a variety of compounds by the proper manipulation of the support material. The analysis of amino

acids and derivatives and the resolution of enantiomers of amino acids by TLC techniques using a wide variety of adsorbents and impregnating agents, the possibility of obtaining relationships between the chromatographic behaviour and chemical structure and the many practical applications drawn from the literature are described in detail in the following sections.

Adsorbents and Thin Layers

A variety of adsorbents such as silica gel, alumina, polyamide and cellulose are available commercially and are used to make thin layers for TLC. Alumina and silica gel are used with or without a suitable binder such as gypsum or starch. Mixtures of two adsorbents or adsorbents impregnated with certain reagents such as 8-hydroxyquinoline or different metal ions have also been used successfully to improve resolution.

Cellulose layers have several advantages: they are stable, they can be used with various specific reagents and they give reproducible data. They are particularly recommended for quantitative evaluation by densitometry. The drawbacks of cellulose layers are that corrosive reagents cannot be used and the sensitivities of detection reactions of certain amino acids are lower than on silica gel layers.

The best known and most widely used adsorbents for TLC purposes are from Merck, but other products can be used satisfactorily. Pre-coated plates are widely available and increasingly used for the investigation of amino acids and their derivatives. For example, ready-made cellulose layers from Macherey-Nagel (Germany) containing MN cellulose-300 in appropriately bound form are one of the best-known products. Chiralplate from the same firm and Chir from Merck, for the separation of enantiomers of amino acids and their various derivatives, contain a coating of reversed-phase silica gel impregnated with a chiral selector and copper ions. Using home-made thin-layer plates is possible and it is recommended that one should not change the brand of adsorbent during a particular set of experiments.

Application of mixed layers of cellulose and the ion exchanger Amberlite CG-120 and a double layer consisting of a 2 cm band of cellulose + cation exchanger (45 + 5 g) in aqueous CM-cellulose (0.05%), with the remaining portion of the layer prepared from cellulose SF suspension, have also been effectively used. A newly synthesized support named aminoplast comparable with that of starch and cellulose has been reported. Nevertheless, silica gel continues to be the most widely used and successful material.

Preparation of Thin-layer Plates

Most thin-layer work is done on layers prepared from water-based slurries of the adsorbents. Even with the same amount and type of binder, the amount of water used for a given slurry varies with kinds and brands of adsorbents. For example, in the case of cellulose the amount of powder to be mixed with water varies depending on the supplier: Serva, Camag and Whatman recommend the use of 60–80 mL, 65 mL and 25 mL water for 10 g of their cellulose powders, respectively. These slurries may be prepared by shaking a stoppered flask or by homogenizing for a few seconds with a mechanical mixer. On the other hand, for the preparation of an aluminium oxide slurry (acidic, basic or neutral), it is recommended that 35 g of aluminium oxide is used with 40 mL water for spreading equipment, and 6 g of adsorbent in 15 mL ethanol–water (9:1) mixture for pouring directly on to the plate without a spreading apparatus. A slurry of 120 g of alumina G in 110 mL of water has been used successfully to make 1 mm-thick layers for preparative TLC. In general, cellulose powders contain impurities that are soluble in water or organic solvents, and these should be removed by washing the cellulose several times with acetic acid (0.1 mol L^{-1}), methanol and acetone and drying before use. The layer is made by turbo-mixing MN (Machery-Nagel) cellulose 300 (15 g) for 10 min in distilled water (90 mL) and then spreading it to give a 0.25 mm thick layer. The layers are left overnight to dry.

A slurry of silica gel G (50 g) in distilled water (100 mL) is prepared and spread with the help of a Stahl-type applicator on five glass plates of 20×20 cm to obtain 0.5 mm thick layers. The plates are allowed to set properly at room temperature and then dried (activated) in an oven at an appropriate temperature (60–90°C) for 6 h or overnight. The plates are cooled to room temperature before applying the samples.

The same method has been used successfully to prepare plates with silica gel, silica gel polyamide, cellulose and these adsorbents impregnated with a variety of reagents including di-(2-ethylhexyl) orthophosphoric acid (HDEHP), tri-octyl-phosphine oxide (TOPO), 8-hydroxyquinoline, dibenzoyl methane and several metal salts. Brucine and tartaric acid are also mixed in slurries of silica gel as impregnating reagents to resolve enantiomers of amino acids and their PTH derivatives. Mixtures of H_2O–EtOH and other organic solvents can also be used, depending on the nature of the impregnating reagents. Citrate and phosphate buffers have also been used for slurrying silica gel in place of water. It is customary to use 0.25

or 0.50 mm thick layers in activated form, but for preparative purposes 1–2 mm layers are best.

Development of Chromatograms

Standard solutions of amino acids are prepared in a suitable solvent such as 70% EtOH or 0.1 mol L^{-1} HCl in 95% ethanol. These solutions are generally applied as tight spots, 1–2 cm from the bottom of each layer, using a glass capillary or Hamilton syringe. In the beginning a higher concentration, e.g. 500 ng or more, is applied; however, the detection limits are determined for the system developed by repeating the experiment with lower concentrations.

The chromatograms are generally developed in rectangular glass chambers, which should be paper-lined for good chamber saturation and pre-equilibrated for 20–30 min with solvent before use. The time taken depends on several factors such as the nature of the adsorbent, the solvent system and the temperature. The developed chromatograms are dried in an oven between 60 and 100°C, and the cooled plates are usually sprayed with ninhydrin reagent. Heating at 90–100°C for 5–10 min produces blue to purple zones of all amino acids except proline (yellow spot).

The same method is adopted for both one- and two-dimensional modes. The locating reagent is used after the second run, and a more polar solvent is generally used to develop the chromatogram in the second dimension.

Separation of Amino Acids

Silica gel and cellulose are the commonest adsorbents for one- or two-dimensional resolution of amino acids. These have been used as such (untreated) or impregnated with some other reagent employing a large number of solvents. Some of the successful systems for one- and two-dimensional resolution of amino acids are given in **Table 1** and **Table 2**. **Table 3** shows a comparative account of the separation of amino acids (hR_F values) on silica gel, cellulose and ion exchange thin layers using *n*-butanol–acetic acid–water (3 : 1 : 1). The data are of great value for separating and detecting amino acids by one-dimensional TLC and based on it the amino acids have been grouped for the separation of 18-component mixtures (separation I) and essential amino acid mixtures (separation II) by calculating

Table 1 Solvent systems for TLC of amino acids on silica gel

Solvent system	*Ratio v/v*
Silica gel	
96% Ethanol–water	7 : 3
n-Propanol–water	7 : 3
n-Butanol–acetic acid–water	4 : 1 : 1
n-Propanol–34% NH_4OH	7 : 3
n-Propanol–water	1 : 1
Phenol–water	3 : 1
Propan-2-ol–water	7 : 3
Butyl acetate–methanol–acetic acid–pyridine	20 : 20 : 5: 5
n-Butanol–formic acid–ethanol	3 : 1 : 1
n-Butanol–acetic acid–chloroform	3 : 1 : 1
n-BuOH–HOAc–EtOAc–H_2O	50 : 20 : 30 : 20
n-Propanol–H_2O	7 : 3
n-BuOH–H_2O–HOAc	40 : 7 : 5
Cellulose	
Propan-2-ol–butanone–1 mol L^{-1} HCl	60 : 15 : 25
2-Methylpropan-2-ol–butanone–acetone–methanol	20 : 1 : 14 : 5
Butanol–acetic acid–H_2O	4 : 1 : 5
Methanol–H_2O–pyridine	20 : 5 : 1
Propanol–8.8% NH_3	4 : 1
Chloroform–MeOH–17% NH_3	20 : 20 : 9
Butanol–acetone–Et_2NH–H_2O	10 : 10 : 2 : 5
Phenol–water	3 : 1
Ethyl acetate–pyridine–HOAc–H_2O	5 : 5 : 1 : 3
n-Butanol–acetic acid–H_2O–EtOH	10 : 1 : 3 : 0 : 3 or 4 : 1 : 10 : 1
Ethanol–conc. HCl	30 : 1
n-BuOH–HOAc–H_2O	4 : 1 : 1
Pyridine–acetone–NH_4OH–H_2O	26 : 17 : 5 : 12
Propan-2-ol–formic acid–H_2O	25 : 3 : 2

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Table 2 Solvent systems for two-dimensional TLC

First	*Second*
Silica gel	
n-Butanol–HOAc–H_2O (4 : 1 : 5, v/v, upper phase)	Phenol–water (15 : 1, w/w)
Chloroform–MeOH–17% NH_3 (2 : 2 : 1)	Phenol–H_2O (3 : 1)
n-Butanol–HOAc–H_2O (4 : 1 : 5, upper phase)	$CHCl_3$–MeOH–17% NH_3 (2 : 2 : 1)
Butanone–pyridine–H_2O–HOAc (70 : 15 : 15 : 2)	$CHCl_3$–MeOH–17% NH_3 (2 : 2 : 1)
Cellulose	
Propanol–HCOOH–H_2O (40 : 2 : 10)	*t*-Butanol–methylethyl ketone–0.88 NH_3–H_2O (50 : 30 : 10 : 10, v/v)
Propan-2-ol–butan-2-ol–1 mol L^{-1} HCl (60 : 15 : 25 by vol.)	2-Methyl propanol–butan-2-one–acetone–MeOH–H_2O–(0.88) NH_3 (10 : 4 : 2 : 1 : 3 :1) or 2-methylpropanol–butanone–propanone–methanol–H_2O (40 : 20 : 2 : 1 : 14.5, v/v)

the resolution possibilities of each pair of acids (**Table 4**).

Amino acids chromatographed in the presence of trichloroacetic acid (used in deproteinizing serum samples) show anomalous behaviour, and this interference can be almost completely removed by predevelopment (twice) in ether saturated with formic acid. The migration sequences for the separation of 18 amino acids on reversed-phase thin layers including C_{18} chemically bonded silica gel and on cellulose in *n*-propanol-H_2O (7 : 3, v/v) have generally been found to be the same. Sorbents with ion exchange properties such as diethylaminoethyl (DEAE)–cellulose have also been used as the stationary phase for TLC separation of the main protein amino acids with *n*-butanol–acetic acid–water (5 : 1 : 6, upper phase) and pyridine– water (4 : 1) in one- and two-dimensional modes.

Locating the spots of amino acids After drying the chromatogram it may be viewed under ultraviolet light if the absorbent had a fluorescent indicator, or

Table 3 hR_F ($R_F \times 100$) values for amino acids on different layers

	A	*B*	*C*	*D*	*E*		
					FX_A	FX_B	FX_C
Ala	41.9	29.0	32.4	28.8	50.9	51.2	53.6
Ser	26.9	16.1	26.4	24.1	67.1	64.1	67.1
Tyr	50.0	36.1	49.4	45.9	11.9	13.9	15.5
Glu	34.4	22.6	30.0	28.2	34.5	29.4	30.6
Asp	26.3	14.8	25.3	21.8	71.5	68.2	68.6
Arg	25.6	11.0	12.9	10.0	1.8	2.2	2.2
Gly	29.4	14.8	25.9	23.5	55.6	52.4	53.6
Leu	75.0	63.9	51.8	48.8	21.8	17.8	19.4
Ile	73.1	60.0	49.4	47.1	27.8	22.2	23.3
Try	55.6	36.1	54.1	51.8	1.8	2.2	2.2
Met	41.0	22.5	47.3	43.5	28.0	27.2	25.0
Val	63.1	48.4	43.5	41.2	42.5	35.0	34.4
Lys	18.1	7.1	10.0	7.1	7.5	5.0	5.6
His	20.0	7.1	11.7	7.1	10.6	8.9	10.0
Phe	67.5	54.8	52.4	50.0	14.4	11.1	11.7
Thr	32.5	21.3	30.0	27.6	67.1	60.0	57.2
Cys	6.9	3.2	14.1	7.1	55.9	50.0	57.9
Pro	43.8	33.5	24.1	21.2			
Time for 17 cm (h)	7	11	4.5	7.5	6.5	6	2

A, Baker Flex cellulose sheets; B, Baker Flex microcrystalline cellulose sheets; C, Whatman K6 silica gel plates; D, Whatman high performance silica gel plates; E, Fixion ion exchange sheets (Na^+ form); FX_A, no prior treatment; FX_B, layer pre-equilibrated with equilibration buffer for 16 h; FX_C layer pre-equilibrated as for FX_B but at 45°C. Solvent for A–D, 2-butanol–acetic acid–water (3 : 1 : 1); solvent for E and run buffer, 84 g citric acid + 16 g NaOH + 5.8 g NaCl + 54 g ethylene glycol + 4 mL conc. HCl (pH 3.3); solvent equilibriation buffer, run buffer diluted 30 times (pH 3.8).

Table 4 Group separation of amino acids

System as in Table 3	*Group*	*Amino acids resolved*
A	I	Leu, Phe, Try, Ala, Glu, Ser, Lys, Cys, Tyr
	II	Leu, Phe, Try, Thr, Lys
B	I	Leu, Phe, Tyr, Val, Glu, Asp, Lys
	II	Leu, Phe, Val, Try, Thr, Lys
C	I	Try, Ile, Val, Ala, Ser, Cys, Lys
	II	Try, Ile, Val, Thr, Lys
D	I	Try, Ile, Val, Ser, Glu, Arg, Lys
	II	Try, Ile, Val, Thr, Lys
FX_A	I	Thr, Gly, Val, Glu, Met, Leu, Phe, His, Lys, Arg
	II	Thr, Val, Met, Leu, Phe, His, Lys, Try
FX_B	I	Asp, Thr, Gly, Val, Met, Leu, Thr, His, Lys, Try
	II	Thr, Val, Met, Leu, Phe, His, Lys, Try
FX_C	I	Asp, Thr, Gly, Val, Met, Leu, Thr, His, Lys, Try
	II	Thr, Val, Met, Leu, Phe, His, Lys, Try

Group I, 18-component mixture of amino acids; Group II, Mixture of essential amino acids.

the compounds – such as dansyl amino acids – fluoresce. Solvent fronts indicate regularity of solvent flow. Ninhydrin is the most commonly used reagent for the detection of amino acids, and a very large number of ninhydrin reagent compositions have been reported in the literature. The reagent may be made slightly acidic with a weak acid following the use of an alkaline solvent and vice versa. Constancy of colour formed may be attained by the addition of complex-forming cations (Cu^{2+}, Cd^{2+} or Ca^{2+}) and specific colours may be produced by the addition of bases such as collidine or benzylamine. Some of the ninhydrin compositions and their applications are described below.

1. A solution of ninhydrin (0.2% w/v in acetone) is prepared with the addition of a few drops of collidine or glacial acetic acid. The chromatogram is dipped or sprayed with the solution and dried at 60°C for about 20 min or at 100°C for 5–10 min. Excessive heating causes a dark background. Most amino acids give a violet colour, while aspartic acid gives bluish-red, and proline and hydroxyproline give a yellow colour; the sensitivity limit is 1 μg.
2. Ninhydrin (0.3 g) in *n*-butanol (100 mL) containing acetic acid (3 mL) is sprayed on a dried, solvent-free layer, which is then heated for 30 min at 60°C or for 10 min 110°C. Detection limits range from 0.001 μg for alanine to 0.1 μg for proline and aspartic acid.
3. Ninhydrin (0.3 g), glacial acetic acid (20 mL) and collidine (5 mL) are made up to 100 mL with ethanol or ninhydrin (0.1%, w/v) in acetone–glacial acetic acid–collidine (100 : 30 : 4%).
4. A solution of cadmium acetate (0.5 g) in water (50 mL) and glacial acetic acid (10 mL) is made up (500 mL) with acetone. Portions of this solution are taken and solid ninhydrin is added to give a final concentration of 0.2% w/v. The chromatogram is sprayed and heated at 60°C for 15 min. The results are noted immediately and again after 24 h, at room temperature. Alternatively, the layer is impregnated thoroughly with the reagent and the colours are allowed to develop in the dark at room temperature for 24 h. This reagent gives permanent colours, mainly red but yellow for proline. Sensitivity is 0.5 nmol.
5. Ninhydrin (1.0 g) in absolute ethanol (700 mL), 2,4,6-collidine (29 mL), and acetic acid (210 mL) has been used for spraying on solvent-free cellulose layers. The chromatogram is then dried for 20 min at 90°C.
6. Development of ion exchange resin layers in ninhydrin (1% w/v) in acetone containing collidine (10% w/v) at room temperature for 24 h, or at 70°C for 10 min has also been recommended.
7. Spray of ninhydrin (0.1% or 0.2% w/v in acetone on chromatograms followed by heating at 60 or 90°C for 10–20 min has also been used.
8. Polychromatic reagent consists of firstly, ninhydrin (0.2% w/v) in ethanol (50 mL) + acetic acid (10 mL) + 2,4,5-collidine (2 mL) and secondly, a solution of copper nitrate (1.0% w/v) in absolute ethanol. The two solutions are mixed in a ratio of 50 : 3 before use. Replacement of ethanol by methanol also gives polychromatic amino acid detection by joint application of ninhydrin and primary, secondary or tertiary amines. The layers are first sprayed with diethylamine, dried for 3 min at 110°C, cooled, and then sprayed with 0.2% w/v methanolic ninhydrin and heated for 10 min at 110°C, when the spots of amino acids appear on

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a pale blue background. Use of ninhydrin (0.27 g), isatin (0.13 g), and triethylamine (2 mL) in methanol (100 mL) gives spots of amino acids on a yellow background.

Several other reactions have also been used for the detection of specific amino acids (**Table 5**). Oxalic acid (ethanolic 1.25% w/v), dithio-oxamide (ethanolic-saturated) and dithizone followed by ninhydrin have been used to aid identification and detection of amino acids with various specific colours. Acetylacetone–formaldehyde gives yellow spots under UV light. Using isatin–ninhydrin (5 : 2) in aq. butanol or modifying ninhydrin detection reagent by addition of D-camphor, and various acids improves identification of amino acids. Spraying of layers with 1,3-indanedione or *o*-mercaptobenzoic acid prior to ninhydrin improves sensitivity limits and colour differentiation. 3,5-Dinitrobenzoyl chloride can be used to detect amino acids at a 3–4 μg level, and synchronization of timing is achieved by coupling pneumatic nebulization with optical fibre-based detection in a chemiluminescence TLC system to detect dansyl-amino acids. Chromatograms sprayed with ninhydrin (0.3 g ninhydrin in 100 mL *n*-butanol plus 3 mL of glacial acetic acid), air-dried for 5 s, resprayed and heated in an oven at 110°C for 10 min gives the best sensitivity, stability and colour differentiation compared with different recipes of ninhydrin and fluorescamine sprays.

Separation of Amino Acid Derivatives

Separation and identification of derivatives of amino acids such as dinitrophenyl (DNP), PTH, dansyl and dimethylamino azobenzene isothiocyanate (DABITC), is very important, particularly in the primary structure determination of peptides and proteins. The preparation of PTH, dansyl, and DNP amino acids, and the methods for their identification after separation from the *N*-terminal of peptides and proteins, are available in literature.

PTH Amino Acids

The PTH amino acids are sensitive to light, and optically active derivatives racemize easily. Both manual and automated, and liquid-phase and solid-phase Edman degradation methods (coupling of the NH_2 group of an amino acid at the *N*-terminal end of a polypeptide or a free molecule with phenyl isothiocyanate) are currently used for small and large polypeptides to establish their primary structure. An automated sequencer can deliver several PTH amino acids in 24 h and these are required to be identified rapidly to match the output.

Table 5 Detection reactions for specific amino acids

Amino acid	*Reagent*
Arg	8-Hydroxyquinoline
Arg	α-Naphthol, urea, Br_2
Asp	Ninhydrin, borate solution, HCl
Cys, Met	NaN_3, iodine
Gly	*o*-Phthalaldehyde, KOH
His	Sulfanilic acid
Ser, Thr, Tyr	Sodium metaperiodate, Nessler reagent
Try	*p*-Dimethylaminobenzaldehyde

TLC has been used for the identification of PTH amino acids since Edman and Begg used it in their classical work describing the automatic sequencer. Various TLC systems with different kinds of adsorbents, such as alumina, silica gel and polyamide, have been reported. The results of some TLC systems used for resolution and identification of PTH amino acids are given below.

Two-dimensional TLC has been carried out using plates coated with polyamide containing three fluorescent additives when all PTH amino acids show coloured spots under UV light. About 0.1 nmol of PTH amino acid can be detected. Typical results are given in **Table 6**. A compilation of solvent mixtures useful in the TLC of PTH amino acids on various supports is given in **Table 7**.

Resolution and identification of PTH amino acids on silica or polyamide layers, as discussed above, do not discriminate between derivatives of Leu/Ile and cannot resolve complex mixtures without two-dimensional chromatography. Difficulties in resolving combinations of PTH Phe/Val/Met/Thr and PTH Asp and Glu are also observed. Use of chloroform–acetic acid (27 : 3, v/v) and chloroform–methanol (30 : 4, v/v) has been found to be extremely satisfactory for discriminating between PTH Asp and PTH Glu, as the difference in their hR_F values is around 10 units. The difficulties in resolving and identifying various combinations of PTH amino acids can be overcome by the use of certain solvent systems, given in **Table 7**.

Detection of PTH amino acids The methods of detection include firstly, spraying a dilute solution of fluorescein on a plain layer of silica gel when the spots are visible as dark areas against a yellow background in UV light; secondly, exposing the dried chromatograms to iodine vapours to locate the spots as light brown compact zones; and thirdly, use of iodine–azide solution when bleached spots on a light brown background are observed. The iodine azide method is considered less sensitive and causes difficulties in demarcating the exact spots and measuring the

Table 6 Characteristic colours of PTH amino acids on polyamide plates containing mixed fluorescent additive 3

PTH amino acid	*Colour after*	
	Second treatment	*Alkaline treatment*
Valine	Red	Red
Proline	Red	Red
Alanine	Red	Red
Glycine[a]	Red	Brownish red
Serine	Red	Brownish red (blue)
Asparagine[a]	Red	Greenish brown (bluish green)[b]
Aspartic acid	Red	Brownish red (dark brown)
Methionine[a]	Red	Brownish red
Leucine	Red	Brownish red
Isoleucine	Red	Red
Lysine	Red	Red
Tyrosine[a]	Red	Red (bluish green)[b]
Threonine[a]	Red	Bluish green (blue)
Glutamine[a]	Red	Greenish brown (white yellow)
Glutamic acid	Red	Red
Phenylalanine[a]	Red	Greenish red (white blue)[b]
Tryptophan[a]	Red	Greenish red (white blue)[b]
Histidine[a]	Red	Blue (light blue)[b]
Arginine[a]	Red	Purple (blue)[b]
Cysteic acid	Red	Brownish red (dark brown)

[a]Spots appear yellow, except glycine (pink); [b]Fluorescent. Solvents: toulene-*n*–pentane–acetic acid (6 : 3 : 2, v/v) and acetic acid–water (1 : 3, v/v) for first and second dimension, respectively. Alkanine treatment: spray 0.05 mol L^{-1} NaOH in methanol–water (1 : 1, v/v), heating at 150°C for 30 min, UV.

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correct R_F. Characteristic changes in the colours of some derivatives are observed by heating the plate after spraying with an alkaline solution when the plate with mixed fluorescent additives is used (**Table 6**). A rapid colour-coded system due to ninhydrin spray is mentioned in **Table 8**; the colours produced allow easy identification of those amino acids that have nearly identical R_F values, for example, Lys and Ser degradation products, Ala/Met/Phe, and Tyr/Thr. The method is significant because it gives positive identifications of PTH Ser/Lys/Glu/Asp and their respective amides, which cannot be identified by gas chromatography (GC).

Table 7 Various solvent systems for TLC of PTH amino acids

	Ratio
Polyamide	
n-Heptane-*n*-BuOH–HOAc	40 : 30 : 9
Toluene–*n*-pentane–HOAc	60 : 30 : 35
Ethylene chloride–HOAc	90 : 16
Toluene–*n*-pentane–HOAc	60 : 30 : 35
EtOAc–*n*-BuOH–HOAc	35 : 10 : 1
n-BuOH–MeOH–HOAc (+ 30 mg butyl fluorescent reagent per litre)	19 : 20 : 1
Silica gel	
Heptane–CH_2Cl_2–propionic acid	45 : 25 : 30
Xylene–MeOH	80 : 10
$CHCl_3$–EtOH and	98 : 2
$CHCl_3$–EtOH–MeOH (in the same direction)	89.25 : 0.75 : 10
$CHCl_3$–*n*-butyl acetate	90 : 10
Diisopropyl ether–EtOH	95 : 5
CH_2Cl_2–EtOH–HOAc (or on cellulose)	90 : 8 : 2
Petroleum ether (60–80°)–acetic acid	25 : 3
n-Hexane–*n*-butanol	29 : 1
n-Hexane–*n*-butyl acetate	4 : 1
Pyridine–benzene	2.5 : 20
MeOH–CCl_4	1 : 20
Acetone–dichloromethane	0.3 : 8

Dansyl Amino Acids

Derivatization of free amino group of amino acids with 5-methylaminonapthalene-1-sulfonyl (dansyl) chloride has become increasingly popular for *N*-terminal determinations in proteins and for manual Edman degradation. In addition, dansylation has also been used as one of the most sensitive methods for quantitative amino acid analysis.

Two-dimensional TLC on polyamide sheets using water–formic acid (200 : 3, v/v) for the first-direction run and benzene–acetic acid (9 : 1, v/v) for development at right angles to the first run has mostly been employed in conjunction with the Edman dansyl technique for sequencing peptides. These solvents cannot resolve Dns-Glu/Asp, Dns-Thr/Ser, and α-Dns-Lys/ε-Dns-Lys/Arg/His. However, a third run in ethyl

Table 8 Characteristic colours of PTH amino acids following ninhydrin application

PTH derivative	*Colour properties*	*NH_4OH colour change*
Proline	UV, colourless	Light blue after heating
Alanine	Purple	Deeper colour
Glycine	Orange	
Serine	UV, purple	
Serine breakdown	Faint orange	Weak red
Asparagine	Yellow	More intense
Carboxymethylcysteine	UV, purple	
Methioninesulfone	Light tan	
Methionine	Faint tan	
Lysine	Very faint pink	Weak blue after heating
Tyrosine	UV, yellow before spray	Intense yellow
Threonine	Colourless	Light tan
Glutamine	Dark green	Dark blue
Phenylalanine	UV, colourless	Faint yellow
Tryptophan	UV, yellow before spray	Deep yellow
Aspartic acid	UV, pink	Darker
Glutamic acid	Grey	Dark blue

Silica gel plates, without fluorescent indicator, developed in heptane–CH_2Cl_2–propionic acid (45 : 25 : 30) and xylene–MeOH (80 : 10), sprayed with iodine–azide and 1.7% ninhydrin in MeOH–collidine–HOAc (15 : 2 : 5), heated at 90°C for 20 min; colour changes by blowing a saturated ammonia atmosphere over ninhydrin plate.

acetate–acetic acid–methanol (20 : 1 : 1) in the direction of solvent 2 resolves Dns-Glu/Asp, and Dns-Thr/Ser. A further run in the direction of solvent 2 and 3 using 0.05 mol L^{-1} trisodium phosphate–ethanol (3 : 1, v/v) resolves the monosubstituted basic Dns amino acids. Use of molarity ammonia–ethanol (1 : 1, v/v) as a third solvent for two-dimensional chromatograms, for the separation of basic dansyl amino acids in particular, has been effective. Most of the TLC systems reported up to 1978 required more than two runs for complete resolution of all Dns amino acids. A few solvent systems to yield separations of basic, acidic and hydroxyl derivatives in the presence of other amino acids without resorting to the third solvent system and R_F values are given in **Table 9**. Additionally, a large number of solvent systems for one- or two-dimensional resolution of dansyl amino acids on silica gel or polyamide have been summarized in **Table 10**. Bhushan and Reddy reviewed the TLC of dansyl, and DNP amino acids and evolved several successful and effective solvent systems for the resolution of almost all the dansyl amino acids on silica gel plates (**Tables 11** and **12**).

Detection of dansyl amino acids In all cases, dansyl amino acids, being fluorescent, have been detected under a UV lamp (254 nm).

DABITC Derivatives of Amino Acids

DABITC reacts with the NH_2-terminal end of an amino acid in basic media to give a 4-dimethylamino azobenzene thiohydantoin (DABTH) amino acid via a DABTC derivative, in a manner similar to the Edman method, where a PTH amino acid is obtained by the reaction of phenylisothiocyanate (PITC). The presence of excess free amino acid does not, in any case, interfere with the analysis.

Two-dimensional TLC on polyamide sheets by ascending solvent flow is used to identify all DABTH amino acids except DABTH-Ile/Leu. No phase equilibrium is necessary; H_2O–acetic acid (2 : 1, v/v) is used for the first dimension and toulene–*n*-hexane–acetic acid (2 : 1 : 1, v/v) is used for the second. For discrimination between DABTH-Ile/Leu, one-dimensional separation on polyamide using formic acid–ethanol (10 : 9, v/v) or one-dimensional separation on silica gel (Merck) using chloroform–ethanol (100 : 3, v/v) is carried out. The successful identification of DABTH amino acids relies on skilful running of the small polyamide sheet and interpretation of the pattern of spots.

Detection of DABITC derivatives of amino acids The use of DABITC reagent during amino acid sequencing of proteins has distinct advantages over the use of dansyl chloride; for example, the colour difference between DABITC, DABTC derivatives and DABTH-amino acids greatly facilitates direct visualization and identification. DABTH amino acids are coloured compounds having absorption maxima at 520 nm in acid media ($\varepsilon = 47\,000$). Thus, using the visible region, the quantitation and

Table 9 R_F values for Dns amino acids in various solvent systems on polyamide sheets

Dns amino acid	R_F in solvent systems									
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
1. Ala	0.53	0.48	0.49	0.69	0.69	0.57	0.81	0.68	0.43	0.76
2. Arg	0.05	0.03	0.03	0.91	0.39	0.09	0.76	0.22	0.01	0.06
3. Asp	0.08	0.07	0.10	0.69	0.38	0.10	0.88	0.37	0.12	0.19
4. Cys	0.03	0.03	0.04	0.19	0.43	0.22	0.78	0.09	0.03	0.06
5. Glu	0.15	0.10	0.15	0.66	0.88	0.02	0.88	0.34	0.05	0.30
6. Gly	0.32	0.21	0.32	0.69	0.63	0.48	0.80	0.48	0.28	0.69
7. His	0.07	0.05	0.13	0.96	0.76	0.32	0.84	0.36	0.06	0.18
8. Ile	0.77	0.54	0.65	0.40	0.57	0.71	0.78	0.76	0.60	0.84
9. Leu	0.70	0.49	0.59	0.34	0.57	0.71	0.78	0.75	0.54	0.80
10. Lys (mono)	0.35	0.21	0.38	0.22	0.09	0.63	0.72	0.58	0.09	0.79
11. Lys (di)	0.53	0.37	0.48	0.78	0.69	0.35	0.82	0.40	0.39	0.76
12. Met	0.52	0.36	0.51	0.43	0.59	0.68	0.80	0.62	0.55	0.81
13. Phe	0.57	0.38	0.53	0.31	0.43	0.68	0.77	0.62	0.51	0.81
14. Pro	0.85	0.66	0.71	0.55	0.74	0.46	0.84	0.75	0.69	0.90
15. Ser	0.12	0.07	0.16	0.81	0.71	0.49	0.82	0.42	0.10	0.44
16. Thr	0.15	0.10	0.26	0.81	0.74	0.57	0.82	0.56	0.16	0.56
17. Tyr	0.63	0.47	0.61	0.00	0.00	0.84	0.73	0.65	0.58	0.91
18. Val	0.72	0.56	0.61	0.47	0.67	0.71	0.81	0.80	0.61	0.88
19. Dns-OH	0.00	0.01	0.00	0.51	0.54	0.16	0.74	0.00	0.04	0.04
20. Dns-NH_2	0.51	0.38	0.47	0.71	0.17	0.96	0.49	0.60	0.40	0.91

Solvent systems: A, benzene–acetic acid (9 : 1, v/v) : B, toluene–acetic acid (9 : 1, v/v); C, toluene–ethanol–acetic acid (17 : 1 : 2, v/v) : D, water–formic acid (200 : 3, v/v); E, water–ethanol–ammonium hydroxide (17 : 2 : 1, v/v); F, ethyl acetate–ethanol–ammonium hydroxide (20 : 5 : 1); G, water–ethanol–ammonium hydroxide (14 : 15 : 1, v/v); H, *n*-heptane–*n*-butanol–acetic acid (3 : 3 : 1, v/v); I, chlorobenzene–acetic acid (9 : 1, v/v); J, ethyl acetate–methanol–acetic acid (20 : 1 : 1).

Table 10 Various solvent systems for TLC of dansyl amino acids

	Solvent systems	*Ratio*
1.	HCOOH	1.5%
	Benzene–acetic acid	9 : 1
2.	Formic acid	1.5%
	Benzene–acetic acid	4.5 : 1
3.	H_2O–pyridine–HCOOH	93 : 35 : 3.5
	Benzene–acetic acid	4.5 : 1
4.	$NH_4Cl + NH_3$ + ethanol	80 g + 22 mL + 10 mL
	Benzene–pyridine–HOAc	75 : 2 : 6
5.	H_2O–propanol–formic acid	100 : 5 : 2
	Benzene–acetic acid	9 : 1
6.	Ethyl acetate–MeOH–HOAc	20 : 1 : 1
	Benzene–HOAc–BuOH	90 : 10 : 5
7.	Formic acid	1.5%
	Benzene–acetic acid	9 : 2
8.	Benzene–anhydrous HOAc, followed by	9 : 1
	EtOAc–MeOH–anhydrous HOAc in the same direction	10 : 1
	Formic acid	1.5%
9.	H_2O–pyridine–HCOOH	93 : 35 : 3.5
	Benzene–acetic acid	4.5 : 1
10.	Formic acid	3%
	Benzene–acetic acid	9 : 1
11.	Me–acetate–*iso*-PrOH–NH_3	9 : 7 : 4
	$CHCl_3$–MeOH–HOAc	15 : 5 : 1
	$CHCl_3$–EtOAc–MeOH–HOAc	45 : 75 : 22.5 : 1
	Pet ether–*t*-BuOH–HOAc	5 : 2 : 2
12.	$CHCl_3$–MeOH	9 : 1
13.	CCl_4–2-methoxyethanol	17 : 3
14.	Benzene–pyridine–acetic acid	80 : 20 : 2

Solvents at serial no. 1–8 : two-dimensional TLC on polyamide layers.
Solvents at serial no. 9–14 : one-dimensional TLC on silica gel layers.

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Table 11 hR_F Values of 10 dansyl amino acids on silica gel thin layers (Sl. no. = serial number)

Sl. no.	*Dansyl amino acid*	*Solvent system*				
		S_1	S_2	S_3	S_4	S_5
1.	Dansyl-L-alanine	62	61	60	50	27
2.	Dansyl-L-isoleucine	80	92	85	85	49
3.	Dansyl-L-leucine	83	85	80	89	65
4.	Dansyl-L-methionine	86	64	62	55	31
5.	Dansyl-L-proline	60	84	72	30	39
6.	*N-O*-dansyl-L-tyrosine	55	73	40	60	18
7.	*N*-α-dansyl-L-tryptophan	51	53	46	40	21
8.	Dansyl-L-phenylalanine	77	76	74	52	40
9.	Dansyl-L-valine	72	88	65	48	35
10.	Dansyl-L-norvaline	75	81	68	45	37

S_1, *n*-heptane–BuOH–HOAc (20 : 8 : 3, v/v); S_2, dichloromethane–MeOH–propionic acid (30 : 1 : 0.5, v/v); S_3, chloroform–HOAc–ethylacetate (24 : 5 : 4, v/v); S_4, chloroform–MeOH–ethyl acetate (23 : 8 : 2, v/v); S_5, chloroform–propionic acid–ethyl acetate (23 : 6 : 4, v/v); R_F values are average of five determinations.

identification of these derivatives become more convenient and sensitive (10 pmol on a polyamide plate). Exposure to HCl vapours turns all yellow spots to red or blue on polyamide sheets.

DNP Amino Acids

Use of DNP amino acids, formed by condensation of 1-fluoro-2,4-dinitrobenzene (FDNB) with the free amino group of an amino acid, was first described by Sanger in 1945, who identified DNP amino acids by paper chromatography. Since then many modifications in the methods of obtaining derivatives of amino acids for sequence analysis and in identification of such derivatives have been reported, and the use of DNP amino acids for sequencing purposes is rapidly going out of practice. Nevertheless, the importance of DNP amino acids has not yet disappeared.

Table 12 hR_F Values of 10 dansylamino acids on silica gel thin layers

Sl. no.	*Dansyl amino acid*	*Solvent system*				
		A_1	A_2	A_3	A_4	A_5
1.	*N*-α-dansyl-L-asparagine	56	75	53	30	35
2.	Dansyl-L-aspartic acid	66	72	60	64	30
3.	α-Dansyl-L-arginine	7	12	3	2	3
4.	*N-N*-didansyl-L-cystine	84	83	68	85	18
5.	Dansyl-L-cysteic acid	82	80	25	15	11
6.	Dansyl-L-glutamic acid	80	90	84	74	55
7.	Dansyl-L-glutamine	62	77	63	41	40
8.	*N*-dansyl-L-lysine	16	20	10	6	8
9.	*N*-dansyl-L-serine	72	85	72	58	32
10.	Dansyl-L-threonine	76	88	76	68	45

A_1, Dichlormethane–MeOH–propionic acid (21 : 3 : 2, v/v); A_2, ethyl acetate–MeOH–propionic acid (22 : 10 : 3, v/v); A_3, chloroform–MeOH–HOAc (28 : 4 : 2, v/v); A_4, chloroform–acetone–HOAc (20 : 8 : 4, v/v); A_5, chloroform–acetone–propionic acid (24 : 10 : 5, v/v) R_F values are the average of five determinations.

Table 13 hR_F Values of DNP amino acids on silica gel thin layers

Sl. no.	N-*DNP-L-amino acid*	*Solvent system*				
		S_1	S_2	S_3	S_4	S_5
1.	Phenylalanine	53	48	85	70	55
2.	Isoleucine	68	82	96	97	60
3.	Tyrosine	25	30	60	52	36
4.	Alanine	40	36	68	50	42
5.	Glycine	28	17	35	25	27
6.	Leucine	65	73	93	90	52
7.	Tryptophan	48	33	53	47	34
8.	Methionine	45	40	75	57	42
9.	Valine	62	65	90	85	47
10.	Proline	41	45	74	60	38
11.	Norvaline	61	62	88	83	45
		Solvent system				
		A_1	A_2	A_3	A_4	A_5
12.	*N*-DNP-L-serine	51	68	70	70	70
13.	*N*-DNP-lysine	21	26	11	7	27
14.	*N-S*-di-DNP-L-cysteine	82	87	77	85	85
15.	*N*-DNP-L-glutamic acid cyclohexyl-amine salt	67	80	83	92	82
16.	*N*-DNP-L-aspartic acid	38	70	75	60	60
17.	*N*-DNP-L-asparagine	30	64	45	38	55
18.	*N*-DNP-L-arginine	10	6	5	3	18
19.	*N,N*-di-DNP-L-cystine	48	70	55	65	82

S_1, *n*-heptane-*n*-butanol-acetic acid (20 : 4 : 1, v/v); S_2, chloroform–propionic acid (26 : 2, v/v); S_3, chloroform–acetic acid (21 : 1, v/v); S_4, chloroform–ethanol–propionic acid (30 : 2 : 1, v/v); S_5, benzene–*n*-butanol–acetic acid (34 : 1 : 1, v/v); A_1, chloroform–methanol–acetic acid (25 : 5 : 1, v/v); A_2, chloroform–propionic acid–methanol (15 : 10 : 1, v/v); A_3, *n*-heptane–butanol–acetic acid (16 : 8 : 4, v/v); A_4, *n*-butanol–ethyl acetate–acetic acid (20 : 8 : 2, v/v); A_5, *n*-butanol–methanol–propionic acid (18 : 8 : 2, v/v). R_F values are average of five determinations.

Kirchner presented considerable information on the analysis of DNP amino acids based on the literature available up to 1970. In one of the earlier methods, thin-layer plates ($20 \times 20\,\text{cm} \times 0.25\,\text{mm}$) were prepared from a mixture of 10 g of cellulose MN-300 and 4 g silica gel H (Merck), homogenized in 80 mL of water, dried overnight at 37°C and developed in the first dimension with two solvents successively: *iso*-propanol–acetic acid–H_2O (75 : 10 : 15) for 15 min and *n*-butanol–0.15 mol L^{-1} ammonium hydroxide (1 : 1, upper phase). The dried chromatograms were developed in 1.5 mol

L^{-1} sodium phosphate buffer (pH 6.0) in the second dimension.

In almost all methods reported, the separation has been carried out in groups of water-soluble and ether-soluble DNP amino acids, and for each group mostly two-dimensional TLC has been performed. A few solvent systems for one-dimensional resolution of DNP amino acids on silica gel plates are shown in **Table 13**.

Detection of DNP amino acids The DNP amino acids have been visualized by UV light (360 nm with dried plates, or 254 nm with wet ones) or by their yellow colour, which deepens upon exposure to ammonia vapours. Thin layers of silica gel usually give an intense purple fluorescence for DNP amino acids under UV light, which masks the presence of very faint spots and decreases the colour contrasts. The cellulose–silica mixed layer gives much lower fluorescence and preserves the colour contrasts between various derivatives. Because of the photosensitivity of these derivatives, it is advisable to carry out their preparation and chromatography in the absence of direct illumination.

Resolution of Amino Acids and Derivatives on Impregnated Layers

The technique of incorporating a suitable reagent with the adsorbent, prior to applying the samples to the plates, originated from simple TLC and can be termed impregnated TLC. The reagents and methods used for impregnation are not to be confused with locating/spray reagents because the latter are required for the purpose of identification even on impregnated plates.

Methods for Impregnation

Of the various methods used for impregnation, one is mixing of the impregnating reagent with the inert support. A second approach is the immersion of the plates into an appropriate solution of the impregnating reagent carefully and slowly so as not to disturb the thin layer. Alternatively, a solution of the impregnating material is allowed to ascend or descend the plate in the normal manner of development; this method is less likely to damage the thin layer. Exposing the layers to the vapours of the impregnating reagent or spraying the impregnating reagent (or its solution) on to the plate have also been employed; spraying provides a less uniform dispersion than the other methods. Another approach is to have a chemical reaction between the inert support and a suitable reagent: the support is chemically modified before making the plate, the compounds of interest are bonded to the reactive groups of the layer.

The impregnating agent participates in various mechanisms in the resolution process, including ion-pairing, complex formation, ligand exchange, coordination bonds, charge transfer, ion exchange and hydrogen bonding.

Amino acids Resolution of amino acids has been reported to be very rapid and improved by using copper sulfate, halide ions, zinc, cadmium and mercury salts, and alkaline earth metal hydroxides as impregnating materials and some of the results are described in **Tables 14–17**. The chromatograms developed in these systems provide compact spots, without lateral drifting of the solvent front. C_{18} layers impregnated with dodecylbenzene sulfonic acid are helpful in confirming the presence of an unknown amino acid in a sample and the migration sequence on these impregnated plates is reversed, probably due to an ion exchange mechanism. Separation of α-amino acids with butan-1-ol–acetic acid–water (3 : 1 : 1, v/v), butan-1-ol–acetic acid–chloroform (3 : 1 : 1, v/v), and butan-1-ol–acetic acid–ethyl acetate (3 : 1 : 1, v/v), on plain and nickel chloride impregnated plates has been reported; the partition and adsorption coefficients for the amino acids under study were determined on both untreated and Ni^{2+} impregnated silica gel in a batch process and correlations were drawn between TLC separation of amino acids on the impregnated gel with adsorption/partition characteristics. The results indicate a predominant role of partitioning in the separation. Application of antimony (V) phosphate–silica gel plates in different aqueous, nonaqueous and mixed solvent systems has also been reported. Some impregnated TLC systems for resolution of amino acids are summarized in **Table 18**.

PTH amino acids As mentioned above, certain difficulties in resolving or identifying various PTH amino acid combinations have successfully been removed and multicomponent mixtures separated with metal impregnated silica gel layers, while other reagents such as (+)-tartaric acid and (−)-ascorbic acid have been used for the resolution of enantiomeric mixtures. The methods reported provide very effective resolution and compact spots (by exposure to iodine vapours) and can be applied to the identification of unknown PTH amino acid; some of these are given in **Tables 19–21**. Some of the successful solvent systems for TLC of PTH amino acids on impregnated plates are summarized in **Table 22**.

High performance TLC (HPTLC)/overpressured TLC (OPTLC) Improvements in the solid-phase materials for TLC have resulted in an increase in

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Table 14 hR_F of amino acids in presence of halides

Sl. no.	Amino acid	Control plate	Amino acids pretreated with			Plates impregnated with		
			Cl^-	Br^-	I^-	Cl^-	Br^-	I^-
1.	Gly	07	08	09	12	07	08	09
2.	Tyr	30	35	40	47	29	30	31
3.	Pro	12	15	19	22	08	09	10
4.	Thr	15	14	15	19	13	14	16
5.	Cys	22	22	25	27	19	20	22
6.	Leu	32	40	47	50T	50T	55T	60T
7.	Met	23	35	36	37	22	23	24
8.	Ile	30	38	44	44	30	30	31
9.	Ala	15	19	13	16T	16T	16	16
10.	Try	35T	40	50T	53	30	31	34
11.	Phe	36T	41	48	48	365	37	38
12.	Val	19	32	25	29	25	26	26
13.	Asp	08	13	14	15	08	09	10
14.	Ser	09	13T	13	14T	08	08	09
15.	His	01	03	04	05	02	02	02
Time	(min)	50	64	67	67	50	50	50

Solvent system: *n*-butanol–acetic acid–chloroform (3 : 1 : 1, v/v); temperature 25 ± 2°C.
T = tailing.

separation efficiency, sample detectability limits and reduced analysis time. HPTLC can be used with advantage for the separation of PTH amino acids but separation of all 20 common PTH amino acids was not achieved initially. A continuous multiple development on silica gel was able to separate 18 samples and standards simultaneously using five development steps with four changes in mobile-phase and scanning densitometry; typical results are given in **Table 23**. PTH-Leu/Ile/Pro have been identified by HPTLC using multiple wavelength detection. OPLC using chloroform–ethanol–acetic acid (90 : 10 : 2) for polar, and dichloromethane–ethyl acetate (90 : 10) for nonpolar PTH amino acids has been successful in their separation and quantitation; the method is claimed to be superior to HPTLC in having relatively increased migration distance, resulting in the resolution of complex mixtures containing a large number of derivatives. OPTLC and HPTLC on RP-8, RP-18, and home-made ammonium tungstophosphate layers have also been used for the analysis of DNP amino acids.

Table 15 hR_F values for amino acids on copper sulfate and polyamide mixed silica gel plates

Amino acid	A	B	C
L-Leucine (Leu)	65	63	71
D,L-Isoleucine (Ile)	66	72	81
D,L-Tryptophane (Try)	63	68	75
D,L-Methionine (Met)	64	64	72
D,L-Valine (Val)	64	60	77
L-Lysine-HCl (Lys)	16T	12	33
L-Histidine-HCl (His)	22T	20	39
D,L-β-Phenylalanine (Phe)	64	65	82
D,L-Threonine (Thr)	50	51	67
D,L-Alanine (Ala)	46	45	64
D,L-Serine (Ser)	40	43	56
L-Tyrosine (Tyr)	58	61	71
L-Glutamic acid (Glu)	41	48	58
D,L-Aspartic acid (Asp)	28	25	44
L-Arginine HCl (Arg)	24T	19	39
Glycine (Gly)	36	46	49
L-Proline (Pro)	37	36	58
L-Cysteine HCl (Cys)	20T	17	29
D,L-2-Aminobutyric acid (Aba)	51	54	61
L-Ornithine	27T	23	35

The values are average of two or more identical runs, 10 cm in 35 min. A, untreated silica gel plate; B, copper sulfate-impregnated silica gel; C, polyamide mixed silica gel layers; T, tailing Solvent, methanol–butyl acetate–acetic acid–pyridine (20 : 20 : 10 : 5, v/v).

Separation of 18 amino acids on cellulose, silica gel and chemically bonded C_{18} HPTLC plates has been achieved. All of these plates contain a preadsorbent zone except the cellulose. Quantification is carried out by scanning standard and sample zones at 610 nm. hR_F values of amino acid standards on reversed-phase and on normal-phase layers in different solvents are given in **Tables 24** and **25**, respectively.

Resolution of Enantiomeric Mixtures of Amino Acids and Derivatives

The measurement of specific rotation is a common and accepted method for evaluating the enantiomeric

Table 16 hR_F values of 15 amino acids on silica gel impregnated with Zn, Cd and Hg salts

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Thr	25	55	42	41T	35	36	42	33	50	40
Ser	12	38	39	28T	32	29	31T	15	40	31T
Gly	10	35	29	23T	28	25	28	16	35	27T
Lys	03	13	07	05	51	08	05	04	10	05
Ala	30	48	40	31	38	36	38	20	5	35
Tyr	60	60	52	50	48	45	51	62	55	56
Ile	55	67	56	52	50	48	54	50	60	53
Leu	50	65	55	55	52	50	56	47	64	55
Cys	00	00	00	00	00	00	00	00	00	00
Met	45	62	48	48	48	42	48	39	54	45
Glu	18T	43	38	36T	34	27	38T	18	36	34T
Try	57	60	53	51	51	44	54	45	60	47
Phe	54	67	57	55	55	46	57	58	68	52
Val	50	63	45	50	52	42	56	47	57	45
Arg	07	19	13	13	09	11	11	10	15	08

Solvent, butyl acetate–methanol–acetic acid–pyridine (20 : 20 : 5 : 5, v/v). Developing time, 30 min. Detection limit, 10^{-4} mol L^{-1}. Solvent front, 10 cm. A, plain silica gel; B, C, D, 0.5%, 0.2%, 0.1% Zn^{2+}C-impregnated, respectively; E, F, G. 0.5, 0.2, 0.1% Cd^{2+}-impregnated, respectively; H, I, J, 0.5, 0.2, 0.1% Hg^{2+}, respectively. T = tailing.

purity of chiral compounds. The determination of enantiomeric excess (*ee*) values is influenced by the presence of impurities and changes in concentration, solvent and temperature, and requires the $[\alpha]_D$ value for the pure enantiomer. The availability of a reliable optically pure standard depends on the analytical method by which it had been resolved from the enantiomeric or racemic mixture of the compound in question. Though TLC provides a direct method for resolution and analytical control of enantiomeric purity, there are few reports on TLC separation of enantiomers.

In general, the following approaches for the resolution of enantiomers have been used.

Indirect method

This method involves reaction of the enantiomeric mixture with a suitable chriral reagent to make the corresponding diastereomeric derivatives prior to chromatography; the choice of chiral selector is

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Table 17 hR_F values of amino acids on untreated plates and plates impregnated with metal sulfates

Aminoacids	*Unimpregnated plate*	*Plate impregnated with*							
		Mn^{2+}	Fe^{2+}	Co^{2+}	Ni^{2+}	Cu^{2+}	Zn^{2+}	Cd^{2+}	Hg^{2+}
Asp	21	51	54	50	62	58	52	59	64
Glu	25	51	63	55	59	61	54	58	65
Phe	45	64	74	67	72	74	69	68	65
Tyr	46	66	73	68	72	70	72	68	71
Lys	7	15	21	22	18	18	16	32	25
Orn	28	15	23	23	19	23	20	28	T
Arg	30	20	25	30	28	28	25	35	33
Ala	30	50	52	53	60	55	49	58	73
Val	48	60	70	58	71	65	59	65	70
Ser	29	45	57	48	57	52	44	56	48
Hypo	26	42	52	47	48	50	43	57	45
Gly	20	43	58	48	52	40	45	54	55
Leu	50	67	72	69	75	71	74	69	SF
Cys	SF	24	37	32	34	35	30	34	40

T, Tailing; SF, migrates with solvent front. hR_F values are the average of at least five determinations.

Table 18 TLC of amino acids on impregnated silica gel layers

Solvent system	*Ratio (v/v)*	*Impregnation*
iso-Amyl alcohol–H_2O–HOAc	6 : 5 : 3	Pyridinium tungstoarsenate
H_2O–EtOAc–MeOH	64.3 : 5.7 : 30	Silanized silica and triethanol amine. SDS, sodium di-octylsulfonate, dodecyl benzene sulfonic acid
0.1 mol L^{-1} HOAc in aq. 50% MeOH		Dodecyl benzene sulfonic acid
Aq. MeOH + I_2 (KCl or HOAc added)		Ammonium tungstophosphate and dodecyl benzene sulfonic acid
Aq. NH_4NO_3 or HNO_3 or H_2O–HOAc–MeOH (79 : 1 : 20)		Ammonium tungstophosphate
H_2O		Polyamide
H_2O–butanol–anhyd. HOAc	4 : 4 : 2	Kieselguhr or cellulose
n-Butanol–acetic acid–water	4 : 1 : 5	Starch–agar (1 : 1)
Propan-2-ol–EtOAc–acetone–methanol–*n*-pentyl alcohol–aq. 26% NH_3–water in first direction; and Butanol–acetone–propan-2-ol–formic acid–water in second direction	9 : 3 : 3 : 1 : 1 : 3 : 3 18 : 8 : 8 : 3 : 6	Cellulose
1 mol L^{-1} NH_4NO_3–0.1 mol L^{-1} HNO_3		Ammonium tungstophosphate
MeOH–butyl acetate–HOAc–pyridine	4 : 4 : 2 : 1	Copper sulfate and polyamide
n-Butanol–acetic acid–$CHCl_3$	3 : 1 : 1	Cl^-, Br^-, I^-
n-Butanol–acetic acid–ethanol	3 : 1 : 1	Hydroxides of Mg, Ca, Ba, Sr
Butyl acetate–MeOH–HOAc–pyridine	4 : 4 : 1 : 1	Zn^{2+}, Cd^{2+}, Hg^{2+}

limited due to the feasibility of its reaction with the analyte.

Direct method

1. This method uses a chiral stationary phase; it may be due to either natural chirality of the material as such, like cellulose, or some sort of synthesis of the phase.
2. Chiral discriminating agents are added to the mobile phase.
3. A suitable chiral reagent is incorporated, such as acid, base, an organic compound or a metal complex with the adbsorbent during plate making, or at a stage before developing the chromatogram.

DL-amino acids Separation of D,L-tryptophan on a crystalline cellulose-coated plate in 1980 seems to be one of the first TLC reports. Applying the principle of ligand exchange, (2*S*, 4*R*, 2′*RS*)-4-hydroxyl-1-(2′-hydroxy dodecyl)-proline was used as the chiral

Table 19 hR_F values of PTH amino acids on Fe^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} impregnated silica plates

Sl. no.	*PTH-amino acid*	*Alone*	*Fe^{2+}*		*Co^{2+}*		*Ni^{2+}*		*Zn^{2+}*	
			0.2%	*0.3%*	*0.05%*	*0.1%*	*0.1%*	*0.2%*	*0.2%*	*0.3%*
1.	Alanine	60	42	41	57	51	38	40	50	43
2.	Aspartic acid	0	0	0	0	0	0	0	0	0
3.	Glycine	39	26	21	44	38	29	30	32	27
4.	Glutamic acid	0	0	0	0	0	0	0	0	0
5.	Isoleucine	90	84	75	72	90	65	71	81	72
6.	Leucine	95	87	71	82	81	70	76	85	76
7.	Lysine	23	8	6	15	17	7	4	10	8
8.	Methionine	70	54	47	81	62	58	51	57	58
9.	Phenylalanine	75	61	49	77	68	52	55	66	58
10.	Proline	97	89	89	84	76	83	90	96	89
11.	Serine	13	5	5	11	9	11	12	8	5
12.	Tyrosine	96	867	69	68	95	85	78	83	78
13.	Tryptophan	95	91	70	91	97	77	82	88	81
14.	Threonine	86	78	57	94	83	60	63	78	70
15.	Valine	85	75	73	96	79	57	58	76	67

Solvent, chloroform–ethyl acetate (29 : 3, v/v); developing time 35 min; solvent front, 10 cm.

Table 20 hR_F *values of PTH amino acids on untreated plates and plates impregnated with sulfates of Mg, Mn, Fe and Co*

PTH amino acid	*S1 (heptane–butylacetate, 15 + 5)*					*S2 (heptane–propionic acid, 20 + 4)*					*S3 (benzene–ethyl acetate, 15 + 3)*[a]	
	PS_1	M_1	M_2	M_3	M_4	PS_2	M_1	M_2	M_3	M_4	PS_3	M_4
Methionine	28	30	26	32	31	43	45	30	32	35	62	78
Phenylalanine	30	35	29	37	34	50	52	36	38	40	67	80
Tryptophan	63	61	51	60	57	71	67	55	55	57	82	94
Valine	49	46	40	51	47	66	62	52	50	55	73	85
Isoleucine	62	62	50	62	59	77	72	61	60	62	78	65
Tryosine	66	64	53	64	61	80	74	64	64	65	84	89
Threonine	57	52	45	56	53	63	64	53	53	53	72	83
Alanine	23	25	23	26	25	32	34	27	25	29	50	67
Serine	55	55	42	55	51	48	46	38	49	40	70	44
Leucine	69	65	54	63	56	76	71	62	60	67	80	96
Lysine	06	04	02	03	05	06	10	04	06	07	18	35
Glycine	17	15	13	15	15	17	20	15	15	18	37	55
Glutamic acid	04	06	05	06	06	04	04	06	06	05	0	14
Aspartic acid	05	07	06	07	07	05	08	07	07	06	0	22
Proline	44	33	31	34	32	44	42	35	35	45	79	96

[a]Compounds moved to solvent front on plates impregnated with sulfates of Mg, Mn and Fe. PS_1, PS_2, PS_3, untreated plates; M_1, M_2, M_3, M_4, treated with sulfates of Mg, Mn, Fe, and Co, respectively. R_F values are the average of at least five determinations. Developed in 30–40 min at 25°C ± 2°C, and exposed to iodine vapours to locate the spots.

selector to resolve several racemic α-amino acids on reversed-phase 18-TLC plates first immersed (1 min) in a 0.25% copper(II) acetate solution (MeOH–H_2O, 1 : 9, v/v), dried, and then immersed in a 0.8% methanolic solution of the chiral selector (1 min); the results are shown in **Table 26**. Ready-to-use *Chiralplates*® are now marketed by Macherey-Nagel, Duren, Germany, and *Chir*® plates are marketed by Merck, Germany. Resolution of DL-methyl Dopa, and DL-Dopa is very successful on Chiralplates using methanol–H_2O–acetonitrile (50 : 50 : 30, v/v) as the mobile phase and ninhydrin as the detecting reagent (**Figure 1**). The R_F values for L-Dopa and D-Dopa were reported to be 0.47 and 0.61, respectively, and the system is capable of resolving enantiomers in trace amounts, with the lowest level of detection of the D-enantiomer in L-Dopa samples being 0.25%. The resolution of enantiomers of α-substituted α-amino acids, and racemic mixtures of natural and nonnatural amino acids, *N*-methylated and

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Table 21 hR_F Values of PTH amino acids on silica plates impregnated with zinc salts

PTH amino acid	*S1 (heptane–butylacetate, 15+5)*				*S2 (heptane–propionic acid, 20+4)*				*S3 (benzene–ethyl acetate 15+3)*			
	L_1	L_2	L_3	L_4	L_1	L_2	L_3	L_4	L_1	L_2	L_3	L_4
Methionine	17	22	18	25	33	33	29	33	42	57	48	58
Phenylalanine	22	25	25	28	37	38	35	36	47	60	52	60
Tryptophan	36	41	41	51	40	50	40	55	67	74	61	82
Valine	40	35	44	42	52	53	54	52	54	68	64	69
Isoleucine	50	46	55	54	50	62	63	63	62	79	74	74
Tryosine	55	48	59	56	62	64	75	65	64	84	79	78
Threonine	47	40	52	48	53	51	52	56	57	72	72	67
Alanine	23	17	21	22	29	27	23	27	35	44	38	44
Serine	49	45	42	50	38	36	37	39	52	66	60	64
Leucine	55	51	55	59	61	50	67	60	65	81	77	76
Lysine	03	02	03	03	04	05	04	07	6	7	6	8
Glycine	15	10	12	13	16	15	15	15	24	29	25	31
Glutamic acid	0	04	0	04	03	02	02	04	0	0	0	0
Aspartic acid	0	05	0	05	04	03	03	05	0	0	0	0
Proline	38	25	32	30	40	40	40	42	70	77	83	72

L_1, L_2, L_3, L_4 plates impregnated with Cl^-, SO_4^{2-}, CH_3COO^- and PO_4^{3-} of zinc, respectively. Other conditions as in Table 20.

Table 22 TLC of PTH amino acids on impregnated silica gel layers

Solvent system	*Ratio (v/v)*	*Impregnation*[a]
$CHCl_3$–H_2O–EtOAc	28 : 1 : 1	Zn^{2+}, Cd^{2+}, Hg^{2+}
$CHCl_3$–MeOH–Benzene	19 : 1 : 9	
CCl_4–HOAc	19 : 1	
$CHCl_3$–Benzene–EtOAc	25 : 5 : 3	Fe^{2+}, Co^{2+}, Zn^{2+}
$CHCl_3$–EtOAc	29 : 3	Fe^{2+}, Co^{2+}, Ni^{2+}
n-Heptane–*n*-butyl acetate	15 : 5	Cl^-, CH_3COO^-, PO_4^{3-}
n-Heptane–*n*-proprionic acid	20 : 4	of zinc, or SO_4^{2-} of
Benzene–EtOAc		Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}
$CHCl_3$–*n*-butyl acetate	10 : 5	
$CHCl_3$–EtOAc	25 : 2	

[a]Various concentrations of each of the impregnating reagent have been used.

N-formylated amino acids, and various other derivatives of amino acids has also been achieved on Chiralplates; typical results are presented in **Tables 27 and 28**. A novel chiral selector from (1*R*, 3*R*, 5*R*)-azabicyclo-[3,3,0]-octan carboxylic acid has been synthesized and used as a copper(II) complex for the impregnation of reversed-phase 18 plates for ligand exchange TLC separation of amino acids and the results were comparable to those on Chiralplates®.

Chiral selectors such as (−)-brucine and Cu-L-proline complex are used to resolve enantiomers of amino acids (**Table 29**), and (+)-tartaric acid and (−)-ascorbic acid for the resolution of enantiomeric PTH amino acids (**Table 30**). The chiral selectors are mixed with silica gel slurry.

Resolution of trytophans and substituted tryptophans on cellulose layers developed with copper sulfate solutions has shown that excess of Cu^{2+} ions decreases the chiral discrimination of the system, and development with aqueous α-cyclodextrin (1–10%) plus NaCl solutions (0.1, 0.5, 1.0 mol L^{-1}) showed the best results with aqueous 4% α-cyclodextrin–1 mol L^{-1} NaCl solution; the results are comparable to Chiralplate®. It has been observed that chiral effects are essentially additive (for cellulose and α-cyclodextrin) and there is a strong temperature dependence for the chiral separations.

α- and β-cyclodextrins, hydroxypropyl-β-cyclodextrin and bovine serum albumin in the mobile phase have provided enantiomeric separations of amino acids and derivatives. Chiral monohalo-s-triazines have been used for the TLC resolution of DL-amino acids. Racemic dinitropyridyl-, dinitrophenyl- and dinitrobenzoyl amino acids are separated on reversed-phase-TLC plates developed with aqueous-org mobile phases containing bovine serum albumin as a chiral agent.

Dansyl-DL-amino acids Reversed-phase TLC plates from Whatman are developed before application of dansyl amino acids in buffer A (0.3 mol L^{-1} sodium acetate in 40% acetonitrile, and 60% water adjusted to pH 7 by acetic acid). After fan-drying, the plates are immersed in a solution of 8 mmol L^{-1} *N*,*N*-di-*n*-propyl-L-alanine and 4 mmol L^{-1} cupric acetate in 97.5% acetonitrile, 2.5% water for 1 h or overnight and left to dry in air. After applying the samples, the plates are developed in buffer A with or without *N*,*N*-di-*n*-propyl-L-alanine (4 mmol L^{-1}) and cupric acetate (1 mmol L^{-1}) is dissolved in it. The enantiomers are detected by irradiating with UV light (360 nm) to yield fluorescent yellow-green spots. Use of 25% acetonitrile is preferred for glutamic and aspartic acids and serine and threonine derivatives. *N*,*N*-di-*n*-propyl alanine can be prepared by the following procedure: L-alanine (17.8 g) is dissolved in ethanol (200 mL) and 10% palladium on activated charcoal catalyst (3 g) and

Table 23 Optimum experimental conditions for the separation of PTH amino acids by continuous multiple development HPTLC

Step	*Mobile phase*	*Plate length (cm)*	*Time (min)*	*PTH amino acid identified*
1.	CH_2Cl_2	3.5	5	Pro
2.	CH_2Cl_2–propan-2-ol (99 : 1, v/v)	7.5	10	Pro, Leu, Ile, Val, Phe
3.	CH_2Cl_2–propan-2-ol (99 : 1, v/v)	7.5	10	Pro, Leu, Ile, Val, Phe, Met, Ala/Try, Gly, Lys, Tyr, Thr
4.	CH_2Cl_2–propan-2-ol (97 : 3, v/v)	7.5	10	Pro, Met, Lys, Tyr, Thr, Ser, Glu
5.	$C_2H_5COOCH_3$–CH_3CN–CH_3COOH (74.3 : 20 : 0.7, v/v)	7.5	10	Asn, Glu/Gln, Asp, Cm-Cys, His, Arg

Table 24 hR_F Values of amino acid standards on reversed-phase layers

Amino acid	*TLC system*					
	1	*2*	*3*	*4*	*5*	*6*
Aspartic acid	30	59	72	60	83	73
Arginine	28	4	35	28	86	82
Serine	55	36	69	50	82	73
Glycine	50	38	62	45	69	54
Tyrosine	91	77	88	68	77	67
Alanine	78	59	71	63	71	54
Glutamic acid	82	70	86	67	83	69
Proline	56	69	64	40	65	46
Cystine	11	12	39	33	85	84
Methionine	90	74	75	59	75	61
Lysine	31	84	27	24	74	79
Tryprophan	90	77	85	63	72	63
Valine	90	74	75	59	75	61
Threonine	78	52	68	50	72	57
Histidine	21	3	29	23	77	68
Phenylalanine	90	76	83	65	72	61
Leucine	90	77	81	62	75	63
Isoleucine	91	77	81	62	74	61

Layers: 1, 2, Whatman C-18; 3, 5, Merck RP-18; 4, 6, Merck RP-18W. Mobile phases: 1, 3, 4, *n*-Butanol–glacial acetic acid–water (3 : 1 : 1, v/v); 2, 5, 6, *n*-propanol–water (7 : 3, v/v).

propionaldehyde (43 mL) is added. The mixture is hydrogenated for 48 h at 40–50°C at an initial hydrogen pressure of 50 psi; the catalyst is removed using a sintered glass filter and the filtrate is evaporated to dryness. The reaction product (*N*,*N*-di-*n*-propyl-L-alanine) is crystallized from chloroform, and the purity may be confirmed by TLC, and carbon, hydrogen, nitrogen analysis.

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Table 25 hR_F Values of amino acid standards on normal-phase layers

Amino acid	*TLC system*			
	1	*2*	*3*	*4*
Aspartic acid	28	27	26	58
Arginine	18	18	17	2
Serine	26	30	27	40
Glycine	26	32	28	43
Tyrosine	46	58	53	78
Alanine	38	32	32	55
Glutamic acid	69	56	50	64
Proline	45	32	28	50
Cystine	10	11	9	30
Methionine	60	59	51	72
Lysine	15	13	10	4
Tryptophan	55	63	57	82
Valine	60	56	49	68
Threonine	34	32	32	53
Histidine	14	14	11	17
Phenylalanine	68	61	55	80
Leucine	79	61	55	78
Isoleucine	78	59	54	77

Layers: 1, Cellulose; 2, 4, Whatman silica gel; 3, Merck silica gel. Mobile phases: 1, Butan-2-ol–glacial acetic acid–water (3 : 1 : 1, v/v); 2, 3, *n*-butanol–glacial acetic–water (3 : 1 : 1, v/v); 4, *n*-propanol–water (7 : 3, v/v).

Table 26 Enantiomeric resolution of amino acids by TLC

Amino acid	R_F *value (configuration)*		*Mobile phase*
	R	S	
Isoleucine	0.37 (2*R, 3R*)	0.44 (2*S, 3S*)	A
Phenylalanine	0.38	0.45	A
Tyrosine	0.34	0.26	B
Tryptophan	0.39	0.45	A
Proline	0.40	0.59	B
Glutamine	0.53	0.37	A

Development distance: 14 cm; saturated chamber. A, MeOH–water–MeCN (1 : 1 : 4, v/v); B, MeOH–water–MeCN (5 : 5 : 3, v/v).

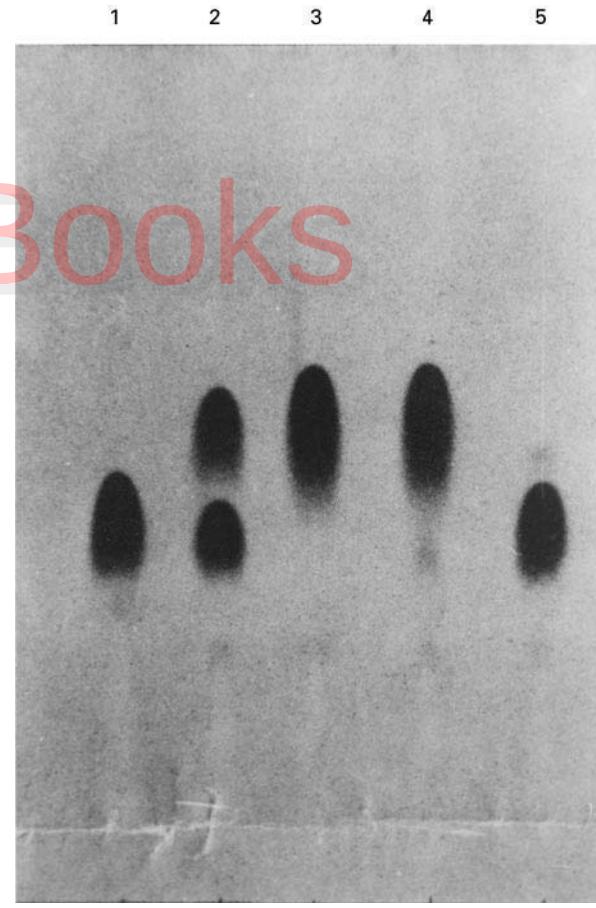

Figure 1 Chromatogram showing separation of different D- and L-dopa samples on Chiralplate. From left to right: 1, L-dopa; 2, D,L-dopa; 3, D-dopa; 4, 3% L-dopa in D-dopa; 5, 3% D-dopa in L-dopa. Developing solvent, methanol–water–acetonitrile (5 : 5 : 3, v/v). Developing time 45–60 min. Detection 0.1% ninhydrin spray reagent.

Table 27 Enantiomeric resolution of α-dialkyl amino acids by TLC

Parent amino acid	R_1	R_2	R_F *value*	*Configuration*	*Mobile phase*
Asp	CH_2CO_2H	CH_3	0.52 (D)	0.56 (L)	A
Glu	$(CH_2)_2CO_2H$	CH_3	0.58 (L)	0.62 (D)	A
Leu	$CH_2CH(CH_3)_2$	CH_3	0.48	0.59	A
Phe	$CH_2C_6H_5$	CH_3	0.53 (L)	0.66 (D)	A
Ser	CH_2OH	CH_3	0.56 (L)	0.67 (D)	B
Try	CH_2-3-indolyl	CH_3	0.54	0.65	A
Tyr	CH_2-(4-OH-C_6H_4)	CH_3	0.63 (D)	0.70 (L)	A
Val	$CH(CH_3)_2$	CH_3	0.51	0.56	A
α-Amino butyric acid	CH_2CH_3	CH_3	0.50	0.60	A
Phe	$CH_2C_6H_5$	CHF_2	0.63	0.70	A
Phe	$CH_2C_6H_5$	CH_2–CH=CH_2	0.57	0.63	A
Phe	$CH_2C_6H_5$	$CH_2CH_2SCH_3$	0.57	0.62	A

Mobile phase: A, methanol–water–acetonitrile (1 : 1 : 4, v/v); B, methanol–water–acetonitrile (5 : 5 : 3, v/v). Development distance 13 cm; saturated chamber.

In a two-dimensional reversed-phase TLC technique for the resolution of complex mixture of dansyl-*dl*-amino acids, the Dns-derivatives are first separated in nonchiral mode using 0.3 mol L^{-1} sodium acetate in H_2O–acetonitrile (80 : 20, pH 6.3) to which 0.3 mol L^{-1} sodium acetate in H_2O–aceto-

Table 28 Enantiomeric resolution of racemates by TLC

Racemate	R_F *value*	*Configuration*	*Mobile phase*
Valine	0.54(D)	0.62(L)	A
Methionine	0.54(D)	0.59(L)	A
Allo-isoleucine	0.51(D)	0.61(L)	A
Norleucine	0.53(D)	0.62(L)	A
2-Aminobutyric acid	0.48	0.52	A
o-Benzylserine	0.54(D)	0.65(L)	A
3-Chloralanine	0.57	0.64	A
S-(2-Chlorobenzyl)-cysteine	0.45	0.58	A
S-(3-Thiabutyl)-cysteine	0.53	0.64	A
S-(2-Thiapropyl)-cysteine	0.53	0.64	A
cis-4-Hydroxyproline	0.41(L)	0.59(D)	A
Phenylglycine	0.57	0.67	A
3-Cyclopentylalanine	0.46	0.56	A
Homophenylalanine	0.49(D)	0.58(L)	A
4-Methoxyphenylalanine	0.52	0.64	A
4-Aminophenylalanine	0.33	0.47	A
4-Bromophenylalanine	0.44	0.58	A
4-Chlorophenylalanine	0.46	0.59	A
2-Fluorophenylalanine	0.55	0.61	A
4-Iodophenylalanine	0.45(D)	0.61(L)	A
4-Nitrophenylalanine	0.52	0.61	A
o-Benzyltyrosine	0.48(D)	0.64(L)	A
3-Flurotyrosine	0.64	0.71	A
4-Methyltryptophan	0.50	0.58	A
5-Methyltryptophan	0.52	0.63	A
6-Methyltryptophan	0.52	0.64	A
7-Methyltryptophan	0.51	0.64	A
5-Bromotryptophan	0.46	0.58	A
5-Methoxytryptophan	0.55	0.66	A
2-(1-Methylcyclopropyl)-glycine	0.49	0.57	A
N-Methylphenylalanine	0.59(D)	0.61(L)	A
N-Formyl-*tert*-leucine	0.48(+)	0.61(−)	A
N-Glycylphenylalanine	0.51(L)	0.57(L)	B

A, Methanol–water–acetonitrile (1 : 1 : 4, v/v); B, methanol–water–acetonitrile (5 : 5 : 3, v/v). Development distance, 13 cm; saturated chamber.

Table 29 Resolution data for enantiomers of amino acids from brucine-impregnated plates

Sl. no.	*D-L-amino acid*	*hR$_F$ pure L*	*D*	*L*
1.	Alanine	53	18	53
2.	γ-Aminobutyric acid			
3.	Isoleucine	35	16	35
4.	DOPA			
5.	Leucine			
6.	Methionine	29	18	29
7.	Norleucine			
8.	Phenylalanine	40	27	40
9.	Serine	50	12	50
10.	Threonine	29	16	29
11.	Tryptophan	31	17	31
12.	Tyrosine	29	22	29

Silica plates impregnated with (−)-brucine, developed in *n*-butanol–acetic acid–chloroform (3 : 1 : 4, v/v), up to 10 cm.

nitrile (70 : 30) is added to give a final acetonitrile concentration of 38% or 47%. For the second dimension, the mobile phase is 8 mol L^{-1} *N*,*N*-di-*n*-propyl-L-alanine and 4 mmol L^{-1} copper(II) acetate dissolved in 0.3 mol L^{-1} sodium acetate in H_2O–acetonitrile (70 : 30, pH 7); the plates are developed in the second dimension using a temperature gradient. The method is reported to be applicable to the resolution of amino acids in a protein hydrolysate with quantitation by densitometry.

β-Cyclodextrin (β-CD) plates have been used successfully for the resolution of enantiomers of dansyl amino acids and β-naphthylamide amino acids. The plates are prepared by mixing 1.5 g of β-CD bonded silica gel in 15 mL of 50% methanol (aqueous) with 0.002 g of binder and acetate in 50/50 MeOH–1% aqueous triethyl ammonium acetate (pH 4.1). Some of the results are shown in **Table 31**.

Table 30 hR_F of pure and resolved enantiomers of PTH amino acids, for tartaric acid-impregnated plate

D,L Mixture of PTH amino acids	*hR$_F$ of pure L*	*D (resolved)*	*L (resolved)*
Met	83	18	83
Phe	85	15	85
Try	95		95
Val	80	21	80
Ile	92	15	92
Tyr	95	16	95
Thr	85	30	85
Ala	55	12	55
Ser	84	10	84

Solvent, chloroform–ethyl acetate–water (28 : 1 : 1, v/v). Development time, 35 min, solvent front, 10 cm, room temperature, 25 ± 1°C. Impregnation with (+)-ascorbic acid resolved D,L mixtures of PTH-Met, Phe, Val, Ala, Ser.

A macrocyclic antibiotic, vancomycin, has been used as a chiral mobile-phase additive for the separation of 6-aminoquinolyl-*N*-hydroxy succinimidyl carbamate (AQC) derivatized amino acids and dansyl amino acids on chemically bonded diphenyl-Freversed-phase plates. Both the nature of stationary phase and the composition of the mobile phase have a strong influence on the enantiomeric resolution; typical results are given in **Table 32**. Another macrocyclic antibiotic, erythromycin, has been used as a chiral impregnating reagent for the resolution of 10 dansyl-DL-amino acids on silica gel plates (**Figure 2**); hR_F values and solvent combinations are shown in **Table 33**. Resolution of dansyl-DL-amino acids has recently been reported (**Table 34**) on thin silica gel plates impregnated with (1*R*, 3*R*, 5*R*)-azabicyclo[3,3,0]octan-3-carboxylic acid, which is an industrial waste material and a proline analogue non-proteinogenic α-amino acid.

Quantitation

TLC is supplemented with spectrophotometric methods for the quantitation of amino acids and their PTH and DNP derivatives.

Amino Acids

The scraped layer corresponding to each spot is extracted with 70% ethanol in a known minimum volume, and ninhydrin reaction is performed followed by spectrophotometry. Six to eight standard dilutions in an appropriate concentration range for each amino acid are prepared; 2 mL of amino acid solution and 2 mL of buffered ninhydrin are mixed in a test tube, heated in a boiling-water bath for 15 min, cooled to room temperature and 3 mL of 50% ethanol is added. The extinction is read at 570 nm (or 440 nm for proline) after 10 min. Standard plots of concentration versus absorbance are drawn for each amino acid. Materials consist of standard solutions of amino acids, acetate buffer (4 mol L^{-1}, pH 5.5), ethanol (50%), methyl cellosolve (ethylene glycol monomethyl ether), and ninhydrin reagent (0.9 g ninhydrin and 0.12 g hydrantin dissolved in 30 mL methyl cellosolve and 10 mL acetate buffer, freshly prepared). The concentration of the unknown sample is read from the standard plots. TLC densitometry can be used to determine 0.5 mg L^{-1} of phenylalanine in blood as an indicator of phenylketonuria.

PTH Amino Acids

The quantitation of PTH amino acids is carried out *in situ* or after elution. For *in situ* determination, the fluorescence-quenching areas of PTH derivatives are usually measured against the fluorescent background

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Table 31 Separation data for enantiomeric compounds on β-CD-bonded-phase plates

Compound, D,L mixture	R_F		*Mobile phase*[a]	*Detection method*
	D	*L*		
Dns-leucine	0.49	0.66	40/66	Fluorescence
Dns-methionine	0.28	0.43	25/75	Fluorescence
Dns-alanine	0.25	0.33	25/75	Fluorescence
Dns-valine	0.31	0.42	25/75	Fluorescence
Alanine-β-naphthylamide	0.16	0.25	30/70	Ninhydrin
Methionine-β-naphthylamide	0.16	0.24	30/70	Ninhydrin

[a]Volume ratio of methanol to 1% triethylammonium acetate (pH 4.1).

at 254 nm. While using a Turner fluorometer fitted with a door for scanning chromatoplates, the position of the scanner, the standardization of time between scanning and the end of chromatography, the loading volume, the developing distance and the layer thickness are the important influencing factors for reproducibility. The quantitation of PTH amino acids is also carried out by measuring their UV absorbance after they have been eluted from the layer. The scraped layer is extracted with methanol overnight, centrifuged for 30 min at 300 rpm, and the spectra of the extracts are recorded in the range from 320 nm to about 230 nm. To obtain reproducible UV absorbances the layers must be washed with methanol prior to development, and with chloroform after the separation has been carried out. The quantitation of PTH amino acids has also been practised as follows: the developed chromatograms are exposed to iodine vapours and the brownish spots scraped off, eluted with 95% ethanol or ethyl acetate, and the iodine removed by warming the sample tubes in a warm-water bath. The optical densities are read at 269 and 245 nm, appropriate blank determinations are carried out, standard plots are drawn, and the concentration of the unknown sample is calculated.

Table 32 RP-TLC enantiomeric separation using vancomycin as chiral mobile-phase additive

Compound	hR_F		*Vancomycin concentration (mol L^{-1})*
	L	*D*	
AQC-allo-*iso*-leucine	14	21	0.025
AQC-methionine	19	23	0.025
AQC-*nor*-leucine	13	16	0.025
AQC-*nor*-valine	21	25	0.025
AQC-valine	23	27	0.025
Dansyl-glumatic acid	21	23	0.04
Dansyl-leucine	03	09	0.04
Dansyl-methionine	05	12	0.04
Dansyl-*nor*-leucine	03	07	0.04
Dansyl-*nor*-valine	05	12	0.04
Dansyl-phenylalanine	03	05	0.04
Dansyl-serine	15	20	0.04
Dansyl-threonine	13	17	0.05
Dansyl-tryptophan	01	03	0.04
Dansyl-valine	06	10	0.04

Mobile phase, acetonitrile–0.6 mol L^{-1} NaCl (2 : 10). Plates developed at room temperature (22°C) in cylindrical glass chambers. Time, 1–3 h for 5 × 20 cm plates. Visualization, UV. AQC, 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, a fluorescent tagging agent; reaction mixture of AQC and amino acid was used without purifying the derivatives.

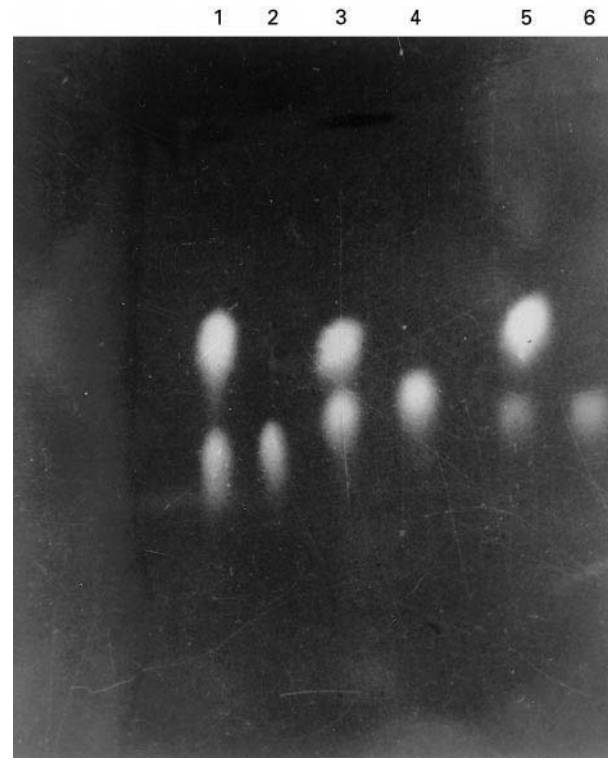

Figure 2 Chromatogram showing resolution of Dns-DL-phenylalanine, valine and leucine. From left to right: 1, Dns-DL-phenylalanine; 2, Dns-L-phenylalanine; 3, Dns-DL-valine; 4, Dns-L-valine; 5, Dns-DL-leucine; 6, Dns-L-leucine. Developing solvent, aq. 0.5 mol L^{-1} sodium chloride + acetonitrile (15 + 1). Developing time 20–25 min. Detection 254 nm.

Table 33 hR_F Values of enantiomers of dansyl amino acids resolved on plates with erythromycin

Dansyl DL-*amino acid*	*Pure* L	*h*R_F *from* DL *mixture*		*Solvent system 0.5 mol* L^{-1} *aq. NaCl–MeCN–MeOH (v/v)*
		D	*L*	
Serine	64	68	64	10 : 4 : 1
	30	36	30	15 : 1 : 1
Glutamic acid	45	56	45	22 : 1 : 0.5
	56	65	56	22 : 1 : 0
	52	59	52	26 : 1 : 0
Phenylalanine	50	65	50	15 : 2 : 0
	20	27	20	15 : 1 : 0
Valine	22	30	22	15 : 1 : 0
Leucine	24	32	24	15 : 1 : 0
Tryptophan	38	47	38	18 : 1 : 0.25
Methionine	56	63	56	25 : 2 : 0.5
Aspartic acid	50	63	50	28 : 1.5 : 0.5
α-Amino-*n*-butyric acid	42	51	42	12 : 1 : 0
Norleucine	63	71	63	16 : 1 : 0 : 0.4 HOAc

Temperature 25 ± 2°C. Solvent front, 10 cm. Time, 20–25 min. Visualization, UV, 254 nm.

DNP Amino Acids

Use of direct fluorimetric quantitation (fluorescence quenching) *in situ* has been recommended. Silica gel G plates are developed in chloroform–benzyl alcohol–acetic acid (70 : 30 : 3 v/v) and *n*-propanol–ammonia (7 : 3 v/v) and polyamide plates are developed in benzene–acetic acid (4 : 1 v/v). The spots are scanned using a Camag/Turner scanner, after being dried in a stream of air, at the scanning speed of 20 mm min^{-1} and an excitation wavelength of 254 nm. Alternatively, the layer is scraped off the plate and extracted for 5 min, with 1 mL 0.05 mol L^{-1} Tris buffer, pH 8.6, at room temperature. Then the slurry is removed by centrifugation and the clear liquid is evaluated by measuring the optical density at 360 nm or at 385 nm for DNP proline. For a blank, a similar extract is obtained from a clear spot on the same layer.

Future Developments

TLC and HPLC are often looked at as competitive methods, but each has its own advantages. In HPLC, finding suitable separation parameters is frequently costly in terms of time and materials; therefore, a combination of the two by first optimizing the particular separation parameter with TLC is a step leading to considerable time-saving and cost for an analysis. TLC is suitable as a pilot technique for the

Table 34 Results from resolution of dansyl DL-amino acids

Dansyl DL-*amino acid*	*Pure* L	*h*R_F *from* DL *mixture*		*Solvent system 0.5 mol* L^{-1} *aq. NaCl–MeCN (v/v)*
		D	*L*	
Phenylalanine	50	65	50	15 + 2
Valine	38	49	38	15 + 1.5
Tryptophan	23	34	23	20 + 0.5
Aspartic acid	55	67	55	15 + 2
	61	70	61	18 + 2
	52	60	52	15 + 1
	30	52	30	20 + 0.5
Leucine	64	68	64	10 + 4 + 1 MeOH
				9 + 3 + 0.5 MeOH
Norvaline	56	61	56	17 + 2 + 0.4 MeOH
				16 + 2 + 0.25 MeOH

Temperature 25 ± 2°C. Solvent front, 10 cm. Time, 25–30 min. Visualization UV, 254 nm.

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investigation of appropriate separation conditions, particularly because with TLC various phase systems can be checked at the same time without expensive apparatus. TLC will continue to serve as a useful method for daily routine control analyses to identify and determine the purity of a variety of compounds, including enantiomers, with ease and speed, and can be readily modified for new situations. A wide choice for separation conditions will always be available as various phase systems can be checked simultaneously.

Further Reading

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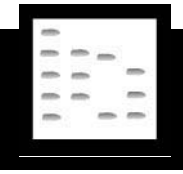
AMINO ACIDS AND DERIVATIVES: CHIRAL SEPARATIONS



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Introduction

It is an interesting feature of life that in general its building blocks, whilst often containing chiral centres, are generally composed from optically pure single enantiomers. An excellent, and well known, example of this is provided by the amino acids as those found in mammals are all of the L-form. This being the case, why is there a need to resolve the enantiomers of amino acids?

The chiral separation of amino acids is important for a number of reasons. Perhaps the major reason for the pharmaceutical industry is the need for optically pure amino acids, of the required configuration, in order to prepare synthetic peptides, both for testing and as potential new drugs. In this case methods are needed to determine optical purity, and measure amounts of the unwanted enantiomer at the 0.1% level and for large-scale isolation for subsequent synthetic work. Another pharmaceutical example is provided by the sulfhydryl drug penicillamine where the D-enantiomer is used to treat arthritis but the L-form is highly toxic, and the optical purity of the drug therefore clearly becomes an issue.

Another interesting reason for wishing to examine the ratio of different amino acid enantiomers is that, as a result of their slow racemization with time, it provides another means of dating archaeological samples. Other applications include the determination of the nature of the amino acids found in microbial peptides and polypeptides where D amino acids are not uncommon (e.g. as constituents of certain antibiotics).

Chiral separations involve the resolution of individual enantiomers that are chemically identical and only differ in the spatial distribution of their individual atoms or groups. As each isomer will contain the same interactive groups, the intermolecular forces involved in their retention will also be the same. Consequently, unless a second retention mechanism is invoked, in addition to those involving intermolecular forces, both enantiomers will exhibit identical retention times on all stationary phases and remain unresolved. A variety of chromatographic separation strategies have been developed to obtain the resolution of amino acids. These include gas, thin-layer and column liquid approaches. In the case of liquid chromatography these methods have